SEARCHING FOR BACTERIA IN STICKY SITUATIONS: METHODS FOR INVESTIGATING BACTERIAL SURVIVAL AT SOLID-AIR INTERFACES INVOLVING WYOMING MX-80 BENTONITE

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Abstract

Effective removal of prokaryotic cells from clay interfaces such as bentonite is essential for quantitative assessment of microbial communities, considering that strong bentonite clay-DNA and –RNA complexes challenge the use of molecular-based techniques. In this study, aerobic bacteria were isolated from Wyoming MX-80 bentonite and sequenced for identification (16S rRNA). A glass-bentonite substrate and sterile bentonite powder were inoculated with *Arthrobacter* sp. (isolated from bentonite) to test cell removal efficiency using sonication and vortexing. Manipulation of pH (pH 7 versus pH 9) did not affect cell removal efficiency, while changes in temperature within limits (15 - 37°C) did affect cell removal efficiency. To evaluate microbial survival during desiccation, bacterial isolates were inoculated onto glass and bentonite-covered glass coverslip substrates, and particulate bentonite. Substrates were desiccated, and cells were removed by vortexing at different time points over 31 days. Abundance of viable cells followed a first-order rate of decrease. Vegetative desiccation-tolerant *Arthrobacter* sp. isolates from bentonite clay had lower loss of viable, culturable cells (0.07 d^{-1} to 0.89 d^{-1}) than did a *Bacillus* sp. isolate (>1 d^{-1}) or a *Pseudomonas stutzeri* isolate (0.79 to >1 d^{-1}), suggesting *Arthrobacter* sp. may be more tolerant of these prolonged periods of desiccation on the bentonite-air interface. Tolerance to matric stress by microorganisms varies depending on the cellular adaptation of the target species, the physical and chemical properties of the given solid-air environment, as well as the employed population and community-based survival mechanisms.

Keywords

adhesion, cell removal, clay interface, desiccation tolerance, nuclear waste storage, nutrient starvation, vegetative cell survival

1. Introduction

A major concern for the future of nuclear power generation is permanent disposal, as well as long-term management of highly radioactive waste generated by the power plants (Kremer et al., 2009). Compacted Wyoming MX-80 bentonite blocks are considered to create low permeable zones, with high sorption capacity for water and low hydraulic conductivity to isolate and seal used nuclear fuel for the Canadian vault design (Johnson et al., 1994; Karnland et al., 2006; NWMO, 2011). An inherent challenge is to evaluate the potential influence of microbial survival and activity on the overall performance and integrity of a vault as part of safety assessment and for designing prediction models. A vault is the near-field engineered excavation consisting of backfill materials, bentonite buffer, and used fuel containers (Wolfaardt and Kober, 2012).

Bentonite clays have been commonly used in civil and hydraulic engineering for containment of waste deposits, for sealing purposes including landfill and foundation dike construction, and in other industries as clarifying and adsorbing agents (Koch, 2002; Montes and Gerard, 2004; Montes et al., 2005). The Wyoming montmorillonite-based clays occur as layers in marine shales and MX-80 material is a blend of various sodium-dominated bentonite horizons (Karnland et al., 2006). Studies suggested that pure bentonite offers sufficient prokaryotic population density reduction when compacted to 2 Mg m^{-3}, reducing water saturation to 26% v/w (Stroes-Gascoyne and Hamon, 2007). The prevailing conditions in vaults using compacted bentonite barriers would be expected to limit prokaryotic population density and activity due to low water activity (a_w of 0.96), high initial temperature, radioactive decay, and constrained spaces due to the small pore size. Considering the hostile conditions, the prime concern for a microbial community is survival and persistence in the bentonite clay's distinct macro- and microenvironment. Thus, compaction of bentonite clay materials impairs microbial mobility and limits diffusional patterns for external water and nutrients. Jalique et al. (2016) studied culturability of microbes in a compacted

bentonite clay plug of uniform density >1.6 g cm^{-3}. Compaction created pore sizes < 0.02 μm and water activity < 0.96, sufficient to suppress microbial growth within the plug over ~8 years. However, culturable bacteria persisted both within the plug's interior and on its surface. Culturable aerobic heterotrophs and nitrate reducing bacteria increased on the bentonite surface over this time, suggesting slow growth and persistence of microbes at the bentonite-water interface. However, clay surfaces may also be hostile environments for bacteria (Biswas et al., 2019). Su et al. (2019) found that when montmorillonite clays were added to a liquid growth medium, bacteria adhered to the clay surface where their numbers and activities declined over time. Desiccation stress at air-bentonite interfaces creates challenges for microbial cells, with low relative humidity (RH) suppressing microbial metabolism (Stone et al., 2016a,b). However, low RH enhanced survival of culturable bacterial cells relative to higher RH environments (75%). Stone et al. (2016a) proposed a concept of whole biofilm resilience promoted by oligotrophy; under low RH conditions, low metabolic activity at the bentonite-air interface could promote prolonged survival of bacteria in a biofilm which could resume activity when more favorable conditions returned. These studies suggest bacteria may survive at bentonite interfaces, potentially including interfaces formed as a result of crack formation due to desiccation of the clay barrier (Stroes-Gascoyne & West, 1997). If the conditions change in the future (e.g. delivery of water), these interfaces are potential hotspots for enhanced microbial activity and sites of vulnerability due to production of metabolic byproducts (again recognizing that wetted clay surfaces can be challenging environments for bacteria). Despite a wealth of information on bacterial survival in aqueous-solid interfaces, very little is known about survival of bacteria under unsaturated solid-air interfaces.

Significant advances have been made in predictive modeling of chemical, thermo-hydrological and physical processes involved in long-term containment of a vault. In contrast, the role of microbial survival and activity and its potential impact requires further assessment considering the complexity of the *in situ*

69 environment and biological processes involved. In order to assess microbial survival at bentonite-air
70 interfaces, effective measures are required to establish a coherent conceptual framework that is suitable for
71 the complexity of microbial interactions with their physical environment. The main purpose of this study
72 was, therefore, to present a suitable method for evaluating microbial survival during desiccation at
73 bentonite-air interfaces using bacteria species that are relevant to an indigenous bentonite community.
74 Here we define desiccation as removal of a substantial amount of water from bacterial cells by matric stress
75 where the cell membrane is exposed to the atmosphere (gas phase). This is in contrast to osmotic stress
76 where the water activity of the cell bathed in an aqueous solution is diminished (Potts, 1994).

77 Adhesion of bacteria to clay surfaces is indirect by production of extracellular polymerase substances
78 (EPS) or macromolecule structures. Bacterial cells are capable of producing surface layers including
79 capsules, fibrils, and polymers that are mainly composed of polysaccharides providing them with glue like
80 properties for attachment and distanced extension from their surface to the surface of the clay (Theng and
81 Orchard, 1995; Potts, 1994). Similarly, adhesion of bacteria and sediment grain surface has been reported
82 and various chemical and or physical techniques are used for removal of bacteria from sediments.
83 Mermillod-Blondin et al. (2001) reported that ultrasonic baths are commonly used for removal of bacteria
84 from sediments before subsequent direct enumeration, and sonication is considered to be an efficient
85 method of removal from sediment particles. Effective bacterial cell removal steps from complex porous
86 solid surfaces are required, in order to transfer and remove bacterial cells effectively for enumeration
87 purposes and survival analysis. Bentonite materials have high capacity for binding to biopolymers such as
88 proteins and nucleic acids, with the strength of this binding dependent on the cation concentration (Na^+ and
89 Mg^{2+}), pH, and the absolute temperature of their medium (Blanton and Barnett, 1969; Fraenkel-Conrat et
90 al., 1969; Lavie and Stotzky, 1986; Lorenz and Wackernagel, 1992; Beall et al., 2009). Mutual sorption
91 between various clays and bacterial cells increases respectively according to: montmorillonite <

92 vermiculite (illite) < kaolinite (in order of decreasing negative charge), while DNA adsorption follows in
93 order of montmorillonite > fine inorganic clay > fine organic clay > kaolinite (Theng et al., 1995; Cai et al.,
94 2006). There is greater emphasis on the use of culture dependent methods for microbial studies involving
95 bentonite clays stemming from the challenges that are present for molecular-based techniques due to
96 formation of strongly bound bentonite clay-DNA complexes. However, the use of these culture dependent
97 methods relies on effective removal of cells from clays. The focus of our paper is primarily on developing
98 effective techniques for removal of bacteria cells from bentonite material, and applying these techniques to
99 examine the survival of bacteria isolated from bentonite under desiccation and nutrient deprivation at the
100 glass-air and bentonite-air interfaces.

101 **2. Experimental section**

102 *2.1 Characterization of cultivable aerobic bacteria from bentonite*

103 *2.1.1 Isolation of cultivable aerobic bacteria from Wyoming MX-80 Bentonite*

104 Aerobic indigenous bentonite bacteria were isolated from commercially purchased Wyoming MX-80
105 bentonite clays (bentonite) (American Colloid Co). A total of 10 isolates were selected based on their
106 morphotypes. From the 10 selected isolates, eight unique colonies were picked from tryptic soy agar
107 (TSA) plates (3 g L^{-1}) (EMD Chemicals Inc., Mississauga, ON, Canada) and two isolates were picked from
108 Reasoner's 2A agar (R2A) (Sigma-Aldrich Canada Co, Oakville, Canada). These plates had been prepared
109 by vortexing 1 g of bentonite in 10 mL of sterile distilled water, spread plating 50 μL of the slurry, and
110 incubating at room temperature for 3 days.

111

112 *2.1.2 Extraction and amplification*

Colonies isolated from bentonite were grown overnight in TSB (3 g L^{-1}) followed by genomic DNA extraction using the MoBio UltraClean Soil DNA Extraction Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) as instructed by manufacturer's protocols. Extracted DNA was stored at -20°C until needed. The 16S rRNA gene of each isolate was amplified for sequencing. Bacteria-specific primers used for the 16S rRNA PCR reaction were forward primer U341 F (5'-CCTACGGGAGGCAGCAG-3') (Muyzer et al., 1993) and reverse primer U803 R (5'-CTACCAGGGTATCTAATCC-3') (Baker et al., 2003). Each PCR reaction totaled 50 µL, containing 1 µL of genomic DNA (~ 50 ng), 25 pmol of each primer, 6.9 µg BSA, 800 µM dNTPs (200 µM of each), Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) with 2.5 units Taq (New England BioLabs, Pickering, ON, Canada) (Yeung et al., 2011). Reaction tubes were kept on ice during the procedure (New England BioLabs, Pickering, ON, Canada). The polymerase chain reaction (PCR) for amplification of DNA was performed (S1000^{TM} Thermal Cycler, Bio-Rad Life Science Group, Canada) with the blocks preheated to the denaturation temperature of 96°C. The thermocycling conditions included the following steps: 1) initial denaturation at 96°C for 1 min; 2) primer annealing, beginning at 65°C in the first cycle and decreasing by 1°C in each of 10 subsequent cycles, with annealing at 55°C in the final 30 cycles; 3) elongation step at 72°C for 3 min. The size of the PCR product for each sample was determined by gel electrophoresis (1% agarose gel with 1.2 µL SYBR® safe DNA stain (Invitrogen, Burlington, ON, Canada)).

2.1.3 DNA Sequencing and Phylogenetic Analysis

DNA sequencing of the PCR products was performed at the Centre for Applied Genomics at SickKids in Toronto with an Applied Biosystems SOLiD 3.0 system. A single consensus sequence was generated from the forward and the reverse nucleotide sequences using BioEdit Sequence Alignment Editor (Version 7.0.9.0; Hall, 1999). The NCBI database of 16S rRNA sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to

136 BLAST search the resulting consensus sequence followed by sequence alignment using Clustal W
137 (http://www.ebi.ac.uk/clustalw/). Construction of the phylogenetic tree was done by a neighbor-joining
138 algorithm with MEGA v5.05.
139
140 *2.2 Removal of bacterial cells from bentonite*
141 In this study, two methods were tested for physical removal of intact cells from clay, ultrasound and
142 vortexing. The purpose was to determine the most effective method for removal and separation of bacterial
143 cells from solid clay particles.
144 Two experimental systems were used to determine cell recovery from bentonite: 1) bentonite sheets on
145 glass coverslips, and 2) dry, particulate bentonite. Bentonite sheets were created by making a slurry of
146 sterile bentonite clay (2.5 MRad irradiation dosage, Department of Chemical Engineering, University of
147 Toronto; 0.2 g/mL in 0.9% sterile NaCl solution), followed by vortexing for 30 min. An aliquot of the
148 slurry (0.5 mL) was spread out on to the surface of a glass coverslip (0.16 mm x 18 mm x 18 mm (VWR
149 International, USA)). All coverslips were placed in a laminar flow ventilation hood to allow complete
150 drying and coverslips with cracked bentonite sheet sections were discarded.
151 *Arthrobacter* sp. (a Gram-positive bacterial strain, isolated from indoor air by Ronan et al., 2013) was
152 used as an inoculum to test methods of cell removal from bentonite sheets or particulate bentonite.
153 *Arthrobacter* sp. were grown in TSB (3 g L^{-1}) for 16-20 hours at room temperature with agitation. The
154 cultures were then washed three times by centrifugation at 8,000 RCF followed by removal of the
155 supernatant and re-suspension of the pellet in sterile distilled water. The washed culture (0.1 mL) was
156 inoculated onto the surface of the glass-bentonite sheets using the large droplet inoculation method (Robine
157 et al., 1998). This method allows rapid and replicable inoculation from a specific volume of the culture
158 onto various substrates (Makison and Swan, 2006; Yazgi et al., 2009; Ronan et al., 2012). After

159 inoculation, the coverslips were allowed to dry by a laminar flow ventilation hood (ambient relative
160 humidity (RH) of 60 ± 5%) for 6 hours. Alternatively, 1 g of dried sterile bentonite clay (2.5 MRad
161 irradiation dosage, Department of Chemical Engineering, University of Toronto) was inoculated with 0.1
162 mL of the washed bacterial suspension. The clay and inoculum were mixed inside a falcon tube using a
163 sterile rod followed by incubation at room temperature for 24 hours.
164 The removal of cells from bentonite sheets or particulate bentonite was performed by sonication and
165 vortexing. Coverslips with bentonite sheets were placed into 50 mL sterile polyethylene tubes with 5 mL of
166 sterile 0.9% NaCl. Particulate bentonite clays were saturated by adding 5 mL of sterile 0.9% NaCl to each
167 falcon tube. Tubes were then exposed to various durations of ultrasound exposure at 40 kHz, various
168 durations of vortexing at 3000 RPM/min, and a sequential combination of both (vortexing first followed by
169 sonication, and sonication as first step followed by vortexing) (Table 1). Cell extractions were done in
170 triplicate for each substrate, treatment, and time combination.
171 The effects of pH and temperature on cell removal were examined using bentonite sheets and particulate
172 bentonite. The 5 mL saline solution in each tube (as above) was adjusted to pH 7 or 9 using HCl (1 N) or
173 NaOH (2 N), and incubated in a water bath at 15, 25, or 37°C for two hours. This was followed by
174 sonication for 20 min to remove cells. Cell extractions were done in triplicate for each temperature, pH,
175 and substrate combination.
176 Cell recovery from bentonite sheets and particulate bentonite was determined by preparation of a serial
177 dilution of the 5 mL solution in each tube, and plating each member of the dilution series onto duplicate
178 TSA plates (3 g L^{-1}) followed by incubation at room temperature for 2-5 days. After incubation, colony
179 forming units were enumerated to determine total cells removed from bentonite.
180

181 *2.3 Investigating the survival of bentonite isolates after desiccation at air-glass and air-bentonite*
182 *interfaces*

183 The survival of bacteria on glass coverslips, dry film from slurry on coverslips, and dry bentonite, as
184 described in previous section, was tested under conditions of desiccation and nutrient deprivation. Strains
185 tested included four *Arthrobacter* sp. isolates from this study (Isolates 2, 3, 4, and 9), the *Bacillus* sp. 1047
186 (Isolate 1), one *Pseudomonas stutzeri* isolate (Isolate 6), and a previously isolated *Arthrobacter* sp. (Ronan
187 et al., 2012). Each bacterium was grown for 16-18 hours in TSB (3 g L-1) at room temperature with
188 agitation. Cultures were centrifuged at 8,000 RCF for five min, pellets were re-suspended in sterile distilled
189 water, repeated three times as above. Bacteria were inoculated onto the glass coverslips, dry film from
190 slurry on coverslips, and dry, sterile bentonite (1 g) using the large droplet inoculation method, as above,
191 transferring 0.1 mL of washed culture to each coverslip or dry bentonite tube. After inoculation, the glass
192 coverslips, dry film from slurry on coverslips, and dry bentonite tubes were allowed to dry by a laminar
193 flow ventilation hood (ambient relative humidity (RH) of 60 ± 5%) for 6 hours.

194 The coverslips were kept in sterile Petri dishes, and dry particulate bentonite (1 g) in sterile tubes inside
195 a humidity chamber (GasPak Chamber, Becton Dickson, Franklin Lakes, NJ, USA), monitored by an
196 Indoor/Outdoor Hygro-Thermometer (Extech Instruments, Waltham, MA, USA). Throughout the
197 experiment, the relative humidity (42 ± 3%) was kept constant using a saturated salt solution (magnesium
198 chloride) prepared as described by Greenspan (1977). The use of saturated salt solutions for investigating
199 survival of microorganisms under desiccation is common for the vapour equilibrium technique (Delage et
200 al., 1998; Saiyouriet et al., 2001; Loiseau, 2001; Montes-H et al., 2003; Tang and Cui, 2007). The vapour
201 equilibrium technique was used to achieve relative humidity of 42 ± 3% for conducting the cell removal
202 experiments in this study (Delage et al., 1998; Saiyouriet et al., 2001; Loiseau, 2001; Montes-H et al.,
203 2003; Tang and Cui, 2007). This technique involves suction ranging from 3 MPa to 1000 MPa depending

204 on the salt solution, and has been recommended for bentonite because of high activity of swelling clays
205 (Tang and Cui, 2007; Tessier, 1984, Romero, 1999, Villar, 2000). This method is advantageous for
206 maintaining a constant value for molar fraction of water in a solution with RH changes between the liquid
207 and gas phase (Tang and Cui, 2007).

208 Sampling was done by using duplicate glass coverslips at each given time point for the glass-air
209 experiments and placing each coverslip in a 50 mL sterile polyethylene tube with 5 mL of sterile 0.9%
210 NaCl and vortexing for 1 min at high speed (3000 RPM/min). The solution was used for preparation of a
211 serial dilution and plating of duplicate samples onto 3 g L^{-1} TSA plates followed by incubation at room
212 temperature for 2-5 days. The number of viable cells at each time point was determined from colony
213 forming units, as above.

214 Triplicate slurry coverslips or dry bentonite (1 g) tubes were used immediately after drying for the
215 bentonite-air experiments and at each time point for enumeration of viable bacteria. Coverslips with
216 bentonite sheets were placed into 50 mL sterile polyethylene tubes with 5 mL of sterile 0.9% NaCl.
217 Particulate bentonite clays were saturated by adding 5 mL of sterile 0.9% NaCl to each falcon tubes. Tubes
218 were vortexed at 3000 RPM/min for 10 min. Cell recovery from bentonite sheets and particulate bentonite
219 was determined by preparation of a serial dilution of the 5 mL solution in each tube, and plating each
220 member of the dilution series onto duplicate TSA plates (3 g L^{-1}) followed by incubation at room
221 temperature for 2-5 days. After incubation, colony forming units were enumerated to determine total cells
222 removed from bentonite.

223 When plotted versus time, the decrease in viable cells were better described by a first-order loss model
224 (exponential decrease) than a zero-order model (linear decrease). Loss of viable cells was then modeled
225 according to:

226 $C_t = C_0 e^{-kt}$

227 Where C_t is cell density at time (t) [days], C_0 is initial cell density, and k is a first-order loss rate [day^{-1}].
228 Viable cell counts (ln transformed) were plotted against time and fit to a linear regression, the slope of
229 which represents k.
230

231 **3. Results and Discussion**

232 *3.1 Isolation and characterization of cultivable aerobic bacteria isolated from bentonite*

233 A total of 10 aerobic bacteria were isolated from commercially purchased MX-80 bentonite clays in order
234 to conduct survival analysis experiments. The phylogenetic analysis based on 16S rRNA gene sequences
235 indicates that all bentonite isolates were closely related to species isolated from similar environments
236 (Figure 1). All identified isolated bacteria in this study, except *Brevibacterium* sp., were previously isolated
237 in other studies involving bentonite barriers for containment of highly radioactive waste. Table 2 provides a
238 brief description of the physiological characteristics of each of the bacteria isolated.

239 Nine out of ten aerobic heterotrophic culturable bacteria isolated based on their distinct morphotypes (to
240 ensure the same strain is not selected twice) from commercially purchased bentonite clays were non-spore
241 forming bacteria. *Bacillus* sp. 1047 was the only isolated endospore forming strain. Despite numerous
242 investigations, the prime concern of most studies in the context of a vault is directed at survival of spore-
243 forming bacteria, especially sulfur reducing bacteria (SRB) due to their potential for microbially-influenced
244 corrosion (MIC) activities. Currently, dormancy is considered as the most common strategy for microbial
245 long-term, high-stress tolerance and resistance during adverse environmental conditions during which
246 bacterial cells remain inactive (Johnson et al., 2007). Numerous investigations demonstrated that some
247 bacterial spores have shown resistance to heat (e.g. Murrell and Scott, 1965; Setlow, 2006; Gomez-Jodar
248 et al., 2015; Marshall et al., 2015), adverse physical conditions (e.g. Pedersen et al., 2000), desiccation (e.g.

249 Setlow, 2006; De benito Armas et al., 2008; Tirumalai and Fox, 2013), radiation (e.g. Tirumalai and Fox,
250 2013; Friedline et al., 2015), and chemical agents (e.g. Pedersen et al., 2000; Leggett et al., 2012; Friedline
251 et al., 2015). Nevertheless, Johnson et al. (2007) reported that maintenance of low-level cellular metabolic
252 activities and DNA repair is essential for sustaining viability over time, adding to the importance of
253 investigating survival of vegetative cells. Bacteria indigenous to bentonite clays and strains introduced
254 during the bentonite block preparation will be present in the vicinity of used spent fuel canisters. Given
255 this, investigations are warranted on survival of non-spore forming bacteria at interfaces formed by
256 cracking as the clay barriers become desiccated. In this study heterotrophic vegetative bacteria, including
257 five *Arthrobacter* sp., two *Brevibacterium* sp., and two *Pseudomonas stutzeri* strains, were previously
258 found in extreme environments and thus, were considered as candidates for further investigation of survival
259 of vegetative bacteria at bentonite-air interfaces.
260
261 *3.2 Removal of bacterial cells from bentonite*
262 Numerous studies reported clay mineral montmorillonite materials interact with bacteria cells by
263 binding to biopolymers such as proteins and nucleic acid (Lorenz and Wackernagel, 1992; Lavie and
264 Stotzky, 1986; Khanna and Stotzky, 1992; Theng and Orchard, 1995; Cui et al., 2006).
265 Bacteria-clay interactions are complex as both the surface of bacterial cells and the surface of crystalline
266 clays are negatively charged and can be defined by both adhesion and sorption of bacteria to the surface of
267 the clays.
268
269 *3.2.1 Removal of bacterial cells adhered to clay*
270 In this study, vortexing and sonication of samples appeared to be successful strategies for removal of
271 desiccated *Arthrobacter* sp. cells from bentonite sheets on glass slides (Figure 2 a, b) or from dry bentonite

particles (Figure 2c, d). Vortexing for 5 min appeared to be sufficient for removal of cells from bentonite sheets, while 10 min was sufficient for removal from particulate bentonite. Sonication of 5 min appeared sufficient for removal of cells, and longer periods did not yield significantly more cells from either bentonite sheets or particles.

A follow-up experiment explicitly compared vortexing, sonication, vortexing followed by sonication, and sonication followed by vortexing to determine which strategy would be most successful for removing viable cells effectively from bentonite prior to enumeration. There was no statistical difference between vortexing (3000 RPM min^{-1}) for 5 min, sonicating (40 kHz) for 20 min, or sonicating (40 kHz) for 20 min followed by vortexing (3000 RPM min^{-1}) for 5 min (Table 3). Unexpectedly, there was a slight decrease in viable cell recovery when bentonite sheets were first vortexed for 5 min followed by sonication for 20 min (one-way ANOVA, using log CFU per mL as dependent variable; removal strategy effect $F_{3,12} = 9.136$, $p = 0.002$). This may be a spurious result, as there was no statistical difference among these same strategies for removal of viable cells from dry, particulate bentonite ($F_{3,12} = 1.686$, $p = 0.223$). Another possible contributor to the slight decrease in viable cell recovery when bentonite sheets were first vortexed prior to sonication is the impact of high shear forces generated on the interface between the glass coverslips and the vortexing fluid leading to dislodging of the cells and increased vulnerability during sonication, potentially leading to harm of viable desiccated cells.

Based on our results, vortexing alone is an acceptable method for removal of bacteria cells, and might serve as a practical method in laboratories that have no access to sonication. In addition, using vortexing alone eliminates potential bias due to optimization of strength and duration of sonication that depends on the type of ultrasonic processors used, including probe-based ultrasonic processors compared to ultrasonic baths. Moreover, it has been reported that Gram-negative bacteria are more susceptible to harm caused by

294 sonication than Gram-positive bacteria (Monsen et al., 2009). This might contribute to misleading
295 conclusions about relative abundance or survival of different bacteria on clays.

296

297 *3.2.2 Removal of bacterial cells sorbed to clay*

298 In contrast to adhesion, sorption involves accumulation of bacteria at the bentonite clay surface and is
299 mainly dependent on the electrolyte concentration, or the pH of the clay matrix. This process could occur
300 by formation of polycationic bridges between cells and the mineral surface, particularly amorphous iron
301 and aluminum hydroxides. Liu et al. (2015) found that adhesion of *Escherichia coli* and *Bacillus subtilis*
302 decreases with increasing pH, while pH is below the point of zero charge for the hydroxide species. Higher
303 adsorption of *Pseudomonas putida* was previously reported by Jiang et al. (2006) to montmorillonite
304 compared to goethite and kaolinite minerals. Additionally, adsorption was reported by their study to be
305 greater for range of temperature 15 to 35°C, and adsorption decreased with increase in pH from 3.0 to 10.0.
306 Different combinations of pH (7 and 9) and temperature (15, 25, and 37°C) were tested to determine
307 their impact on removal of viable cells from bentonite without harming or inducing stress on desiccated
308 *Arthrobacter* sp. cells. The tested temperature range did affect removal of cells from bentonite sheets (on
309 glass coverslips), with recovery of viable cells increasing with temperature (Table 4) (two-way ANOVA
310 using temperature and pH as independent factors, log CFU mL^{-1} as dependent variable; temperature effect
311 $F_{2,12} = 20.036$, $p < 0.001$). There was not, however, a similar temperature effect in dry, particulate
312 bentonite ($F_{2,12} = 0.492$, $p = 0.623$). There was no effect of pH (7 vs. 9) on recovery of viable cells from
313 either bentonite sheets ($F_{1,12} = 3.069$, $p = 0.105$) or dry, particulate bentonite ($F_{1,12} = 0.758$, $p = 0.40$). The
314 absence of a temperature effect in particulate bentonite, or of a pH effect on either particulate bentonite or
315 bentonite sheets may reflect already high recovery rates, regardless of the temperature and pH conditions in

316 those cases; recovery of viable cells at all pH and temperature combinations in particulate bentonite, for
317 example, reflected maximum recovery in the vortex/sonication comparison study (Figure 2).
318
319 *3.3 Survival of bentonite isolates after desiccation at glass-air and bentonite-air interfaces*
320 Isolate 1, identified as *Bacillus* sp. 1047, was an endospore former strain previously reported as resistant
321 to radiation, heat, and desiccation (Table 2). The *Bacillus* sp. had poor survival of viable vegetative
322 (culturable) cells at solid-air interfaces. There remained no viable *Bacillus* sp. vegetative cells (Isolate 1)
323 that could be recovered and cultured on any of the three substrates beyond one day (loss rates of $> 1\ d^{-1}$ or
324 100% loss within 1 d of desiccation) (Table 5). Previous studies reported strong adherence of aerobic soil
325 borne spores to various solid interfaces including clay minerals (kaolinite or bentonite) (Nováková, 1977;
326 Ammann & Brandi, 2011). Considering that the spores of this species were reported as resistant to
327 desiccation, it is not clear if the spores were effectively removed from the glass surface, dry particulate
328 bentonite, and the slurry coverslips since the proposed methods are optimized for removal of vegetative
329 bacteria cells. Aerobic soil-borne spore formers including the *Bacillus* species isolated from bentonite
330 clays and used in this study are found in various soil environments. It is recommended to further
331 investigate the survival of *Bacillus* sp. 1047 spores at solid-air interfaces.
332 The survival of nine non-spore forming vegetative bacteria isolated from bentonite clays was
333 investigated at glass- and bentonite- air interface. Non-spore forming vegetative anhydrobiotic bacterial
334 cells can be defined by their singular deficiency in water, which differs from a cell under osmotic stress or
335 freeze tolerant cells covered in extracellular ice, where their major constituents lack a monolayer of water
336 (Potts, 1994). Drying of bacteria cells at relative humidity (RH) of 40% and 30% leads to cell water
337 content of 0.1 g $H_2O\ g^{-1}$ dry weight and 0.03 g of $H_2O\ g^{-1}$ dry weight, respectively, and this lower limit
338 represents the value measured in anhydrobiotic cell types (Potts, 1994). No viable culturable cells were

339 recovered after drying for Isolate 5 (*Brevibacterium* sp.), Isolate 7 (*P. stutzeri*), Isolate 8 (*Brevibacterium*
340 sp.), or Isolate 10 (*Arthrobacter* sp.), suggesting that the isolated strains are poor survivors under
341 desiccation at solid-air interfaces including at glass-air and bentonite-air interfaces. These isolates were,
342 therefore, not included in Table 5. *Pseudomonas stutzeri* (Isolate 6) had poor survival of viable (culturable)
343 cells at solid-air interfaces and loss of viable cells on all surfaces was rapid for Isolate 6 as well. In contrast
344 *Arthrobacter* sp. isolates had much greater survival, with cell loss rates of 0.04 to 0.89 d^{-1}, Isolate 10
345 notwithstanding. Generally, culturable *Arthrobacter* sp. could be recovered 28 days after desiccation and
346 nutrient starvation. The results obtained from isolated *Arthrobacter* sp. strains from bentonite clays were
347 compared with the strain isolated previously by Ronan et al. (2013) from air. The rate of cell loss for the
348 isolated strain from air was lower at air-glass interface compared to all strains isolated from bentonite
349 clays. However, the decrease in loss of cells per day was higher at bentonite-air interfaces (Bentonite sheet
350 on glass coverslip and particulate bentonite) compared to the strains isolated from bentonite clays,
351 excluding Isolate 10 with no initial counts right after desiccation. The relatively better survival of
352 Arthrobacter under desiccation is consistent with the observation of Stone et al. (2016a) that *Arthrobacter*
353 sp. biofilms were more resilient following desiccation at the bentonite-air interface than were *Pseudomonas*
354 sp. biofilms. While vegetative cells of some species are more resistant to desiccation, there is variation
355 among different strains based on the genotype and phenotype of the bacterial cells. Survival of each
356 bacteria strain could also then depend on population based (and community of microorganisms at large)
357 survival mechanisms. Since the initial population size for each strain varies depending on the associated
358 growth pattern, additional investigations are required to gain insights about population (with consideration
359 of pure culture and mixed community) mediated survival at solid-air interfaces including at MX-80
360 bentonite clay-air interfaces. Furthermore, presence of bentonite clays as the solid substrate did not aid
361 desiccation tolerance of any of the isolated bacteria from bentonite clays.

4. Conclusions

There is growing interest in understanding the mechanisms involved for survival of prokaryotes at solid-air interfaces, such as clay-air interface, to enable prediction of microbial activities, and mitigation of microbial corrosion. Methods presented here demonstrated successful removal of cells from bentonite either by sonication for more than 10 min or by vortexing for more than 5 min. We anticipate tolerance to matric stress by microorganisms will depend on cellular adaptation of the target species, the physical and chemical properties of the given solid-air environment, as well as population and community-based survival mechanisms. Interactions of bacteria cells with clay particles may inhibit their activity, and may do so to different degrees among various bacterial species. Consistent with this expectation, variation among bacterial species in desiccation tolerance of vegetative cells at the bentonite-air interface was evident. The approach used here accounted only for recovery of culturable cells. Future work on survival and recovery of culturable cells from clays could be complemented with scanning electron microscopy to better enumerate cell densities on clay surfaces, total cell removal, and to help differentiate survival of culturable cells from survival of all intact cells (culturable and not).

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574 **Figure Captions**

575 Figure 1. The phylogenetic position of the 10 aerobic bentonite isolates along with the most closely related
576 identified species. The similarity of bentonite isolates is based on the V3-V4 regions as described in the
577 variability map of Van de Peer et al., 1996) of the16S rRNA. The construction of the tree was done by
578 neighbour-joining algorithm with the numbers on the nodes corresponding to the values obtained from
579 bootstrap on 1000 replicates. The out-group was done using Aquifex pyrophilus with scale bar indicating
580 the approximate number of base changes per position of nucleotide sequence.

581 Figure 2. Removal of viable (culturable) *Arthrobacter* sp. cells from a) dried bentonite sheet (on glass
582 coverslips) by vortex; b) dried bentonite sheet by sonication; c) particulate bentonite by vortex; or d)
583 particulate bentonite by sonication at various time intervals, 24 hours after inoculation, under conditions of
584 desiccation and nutrient starvation and incubation at room temperature. Line represents the viable
585 (culturable) cells inoculated onto the bentonite at time zero. Symbols represent mean viable cell counts in
586 media following removal by sonication or vortex. Error bars represent standard deviation.

587 Table 2. Summary of relevant findings on the effect of desiccation on the identified isolated indigenous
588 bacteria from bentonite clays

Isolate Number	Isolate identification	Physiological Characteristics for Stress Tolerance
1	*Bacillus* sp. 1047*	-Endospore formation -Spores resistant to radiation, heat, desiccation and toxic chemicals (de Benito Armas et al., 2008)
2,3,4,9, 10	*Arthrobacter* sp.**	-Non-spore-forming bacteria -Desiccation tolerance -Production of compatible solutes -Lower zone community of rocks in Dry Valley of the Ross Desert, Antarctica with prolonged periods of evaporation and extreme environment (Kappen and Friedmann, 1983)
5,8	*Brevibacterium* sp.	- Non-spore-forming bacteria -Chemoorganotrophic -Cold resistance -Growth in 2% or 5% NaCl medium

		-Selenium tolerance (some strains up to 15.9 mg SeL^{-1})
		(Tong et al., 2014)
		-Lower zone community of rocks in Dry Valley of the Ross Desert, Antarctica with prolonged periods of evaporation and extreme environment (Kappen and Friedmann, 1983)
6,7	*Pseudomonas stutzeri****	- Non-spore-forming bacteria
		-Facultative anaerobe
		- Remarkable physiological and biochemical diversity and flexibility, organotrophy with wide range of organic substances, oxidation of inorganic substrates by chemolithotrophy, resistance to heavy metals, recycling of C,N,S, and P, wide range temperature support (Lalucat et al., 2006)
		-Some members of barotolerant (Kaneko et al., 2000)
		-Wide range of temperature support (Kaneko et al., 2000)

589

590 *Previously described from bentonite by Pedersen et al. (2000) and Chi Fru et al. (2008); **Previously
591 described from bentonite by Chi Fru et al. (2008); ***Previously described from bentonite by Stroes-
592 Gascoyne & West, 1997, Stroes Gascoyne et al., 1997, Pedersen et al. (2000) and Chi Fru et al. (2008).